

## Stereospecificity in the enzymatic hydrolysis of cyclosarin (GF)

Steven P. Harvey<sup>a,\*</sup>, Jan E. Kolakowski<sup>a</sup>, Tu-Chen Cheng<sup>a</sup>, Vipin K. Rastogi<sup>a</sup>,  
Louis P. Reiff<sup>a</sup>, Joseph J. DeFrank<sup>a</sup>, Frank M. Raushel<sup>b</sup>, Craig Hill<sup>b</sup>

<sup>a</sup> U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD 21010-5424, USA

<sup>b</sup> Texas A&M University, Department of Chemistry, P.O. Box 30012, College Station, TX 77843-3012, USA

Received 2 February 2005; received in revised form 31 March 2005; accepted 7 April 2005

### Abstract

Enzymatic catalysis is one means of accelerating the rate of hydrolysis of G-type organophosphorus nerve agents. Here, the stereospecificity of the catalysis of cyclosarin (GF, *O*-cyclohexyl methylphosphonofluoridate) hydrolysis by several enzymes was investigated. Stereospecificity was not evident at 3 mM GF but was evident at 0.5 mM GF. The differential effect was apparently due to fluoride-catalyzed racemization of the substrate. *Alteromonas* sp. JD6.5 organophosphorus acid anhydrolase (OPAA), *Alteromonas haloplanktis* OPAA and the wild-type phosphotriesterase (PTE) enzymes were all found to catalyze preferentially the hydrolysis of the (+)GF isomer, as determined by GC analysis of the remaining unreacted (–)GF isomer. Acetylcholinesterase inhibition experiments showed the purified (–)GF isomer to be approximately twice as toxic as the racemic mixture. One PTE mutant, H254G/H259W/L303T, was found to reverse the native PTE stereospecificity and preferentially catalyze the hydrolysis of the (–)GF isomer, as shown by its complementation of *Alteromonas* sp. JD6.5 OPAA and by GC analysis of the remaining (+)GF isomer. This procedure also permitted the individual preparation of either of the two GF isomers by enzymatic degradation followed by extraction of the remaining isomer.

© 2005 Elsevier Inc. All rights reserved.

**Keywords:** Stereochemistry; Cyclosarin; GF; Enzyme; Isomer; Stereospecificity; Acetylcholinesterase

### 1. Introduction

The organophosphorus acid anhydrolase (OPAA), from *Alteromonas* sp. JD6.5 has been shown to catalyze the hydrolysis of a number of toxic organophosphorus compounds including several G-type chemical nerve agents, the generalized structure of which is shown in Fig. 1 [1,2]. Its gene has been cloned into *Escherichia coli* and the enzyme can be produced at concentrations up to 300 mg per liter of culture, corresponding to approximately 50% of the total cellular protein [3]. The gene encoding a similar OPAA enzyme from *Alteromonas haloplanktis* has also been cloned and expressed in *E. coli* [4].

The phosphotriesterase enzyme (PTE) has some catalytic properties similar to OPAA. In addition, PTE possesses unique catalytic activity against the nerve agent VX (*O*-ethyl-*S*-(2-diisopropylaminoethyl) methylphosphonoth-

ioate. In nature, the PTE gene was found on dissimilar-sized plasmids in two soil bacteria, *Pseudomonas diminuta* MG and *Flavobacterium* sp. ATCC 27551 [5–7]. The gene has been cloned and sequenced [6–8] and the enzyme has been overexpressed in several systems [9–11]. PTE catalyzes the hydrolysis of a broad spectrum of organophosphorus compounds including those with P–O, P–F, P–CN and P–S bonds to their leaving groups [9,12–15]. Recently, a number of site-directed mutants derived from PTE have also been characterized with respect to their activity against various organophosphorus compounds [16–18].

While several reports have described the initial rate kinetics of these enzymes on various chemical nerve agent substrates, relatively little attention has been paid to the stereospecificity of those reactions [3,12,13,15–18]. This issue is important for two reasons: First, it is possible that the differential activity on respective stereoisomers could affect the overall enzymatic detoxification rate (for this reason, it needs to be determined whether all, or at least the most toxic stereoisomers of a particular chemical agent are effective

\* Corresponding author. Tel.: +1 410 4368646; fax: +1 410 6125235.

E-mail address: [steve.harvey@us.army.mil](mailto:steve.harvey@us.army.mil) (S.P. Harvey).

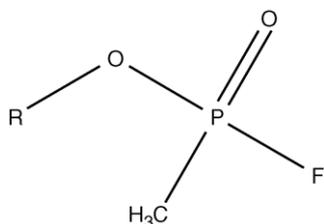


Fig. 1. Generalized structure of G-type chemical nerve agents. R = cyclohexyl for GF.

substrates for these enzymes, and under what conditions), second, it is possible that enzymatic stereospecificity could be exploited to produce higher value products through synthetic routes. Single enantiomer compounds play a critical role as biologically active compounds and hence the stereospecific and substrate-specific nature of enzymes makes them a good choice as catalysts in the synthesis of pharmaceuticals and other fine chemicals.

Chemical nerve agents and enzymes both exert their effects in a biological, chiral environment, so it might be expected that the enzymatic effects on the agents would be stereospecific. This specificity was first reported in 1955 by Michel [19], who observed a biphasic inhibition of acetylcholinesterase (AChE) with GB (*O*-isopropyl methylphosphonofluoridate). It is known that the chemical nerve agents GB, GD (*O*-pinacolyl methylphosphonofluoridate), GA (ethyl *N,N*-dimethylphosphoramidocyanidate) and VX all bind AChE stereospecifically, resulting in a significant difference in toxicity between their respective enantiomers (Table 1). The data from Table 1 can be briefly summarized as follows: For GD, the P(–) isomers are almost solely responsible for the compound's toxicity. For GB, the P(–) isomer is approximately twice as toxic as racemic GB, indicating that essentially all the GB toxicity is associated with the P(–) isomer. For VX, the (–) isomer is approximately 13 times more toxic than the (+) isomer, while the GA (–) isomer is approximately seven times more toxic than the GA (+) isomer.

Table 1  
Toxicity of GB, GD, VX and GA isomers

Compound	Isomer(s)	LD <sub>50</sub> (mouse) (μg/kg)	Reference
GB	P(–)	41	[20]
GB	Racemic	83	[21]
VX	P(–)	12.6	[22]
VX	P(+)	165	[22]
VX	Racemic	20.1	[21]
GD	C(+)/P(–)	99	[23]
GD	C(–)/P(–)	38	[23]
GD	C(+)/P(+)	>5000	[23]
GD	C(–)/P(+)	>2000	[23]
GD	Racemic	156	[23]
GA	P(–)	119	[24]
GA	P(+)	837	[24]
GA	Racemic	308	[24]

In the case of GF, the toxicity of the individual isomers has not to our knowledge been reported. The intravenous toxicity of racemic GF though, has been reported as 53.0 μg/kg (rats), 15.3 μg/kg (rabbits) and 9 μg/kg (goats) [25]. These findings suggest that the rat toxicity of racemic GF is comparable to the mouse toxicity of racemic GB and GD.

The objective of this work was to investigate the stereospecificity of the *Alteromonas* sp. JD6.5 OPAA, *A. haloplanktis* OPAA and wild-type PTE enzymes with GF as their substrate. We also sought to develop a chromatographic means to separate the two GF isomers for analytical purposes, to measure their relative AChE-inhibiting activity, and to specifically produce individual isomer preparations through the enzymatic degradation of the contrasting isomer. Finally, the stereospecific GF activity of a series of site-specific PTE mutants was investigated.

## 2. Materials and methods

### 2.1. Enzymatic assays

Reactions were conducted in a temperature-controlled vessel in a total volume of 2.5 ml. Buffering was provided by 50 mM bis-tris-propane at pH 7.2. MnCl<sub>2</sub> was added to the buffer to a final concentration of 1 mM for OPAA assays. CoCl<sub>2</sub> was added to a final concentration of 1 mM for PTE assays. Fluoride measurements were made with a fluoride electrode attached to a Fisher Accumet 925 meter attached to a computer.

### 2.2. Gas chromatography method for separation of GF isomers

A Hewlett-Packard model 6890 GC equipped with a flame photometric detector in the phosphorus mode and a 25 m × 250 μm i.d. × 0.12 μm Chirasil-Val-L column (Chrompack) was used to analyze the GF stereoisomers. The oven temperature was 90 °C (isothermal) and the inlet and detector temperatures both were 200 °C. The injection volume was 1.0 μl with a 100:1 split ratio. The carrier gas was helium with a 1 ml/min flow rate. Under these conditions the (–)GF and (+)GF peaks were separated by 0.15 min at a retention time of approximately 8 min.

### 2.3. Polarimetry

Specific rotation of the single GF isomer was calculated based on measurements of the observed rotation made at 589 nm (sodium line) using a Perkin Elmer 141 Electronic Polarimeter and a sample cell with a path length of 10 cm.

### 2.4. AChE inhibition assays

AChE enzyme inhibition assays were conducted as described previously [26], with minor modifications. The sub-

strate 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was obtained from Aldrich Chemical Co. (Milwaukee, WI). DTNB was dissolved in 100 mM potassium phosphate buffer at pH 7.0 to a final concentration of 0.01 M. AChE was purchased from Sigma Chemical Co. (St. Louis, MO). Acetylthiocholine (a chromagenic AChE substrate) was purchased from Aldrich as an iodide salt and was 98% pure.

AChE solutions were prepared at 10 ng/ml in water and split into two 10 ml fractions. To one fraction was added 1  $\mu$ l of a  $10^{-9}$  dilution of GF (either racemic or the chromatographically pure single isomer) in isopropanol. The other fraction (uninhibited) received no GF. Aliquots were removed in triplicate from both fractions and analyzed to assess the level of enzymatic activity on acetylthiocholine by spectrophotometric measurements at 405 nm.

### 2.5. Construction of mutant PTE strain

The site-directed mutant PTE strain was constructed using the method of overlap extension [27] and cassette insertion with a synthetic gene [28].

### 2.6. Enzyme preparation

The recombinant *A. haloplanktis* OPAA enzyme was prepared as described by Cheng et al. [29]. Briefly, the cells were grown to mid-log phase in Luria-Bertani (LB) broth and induced for 6 h with IPTG. Ammonium sulfate fractionation (40–65% saturation) yielded ~75% pure enzyme. Further purification through Q-Sepharose yielded an enzyme with 90–95% estimated purity.

The recombinant *Alteromonas* sp. JD6.5 OPAA was prepared according to the method described by Cheng et al. [30]. Briefly, the *E. coli* host cell containing the cloned OPAA gene was grown to late log phase in 11 of LB broth in a bioreactor. Cells were harvested and resuspended in 10 mM bis-tris-propane, pH 7.8. The enzyme was then purified by ammonium sulfate fractionation. The 40–65% ammonium sulfate pellet was redissolved, dialyzed and loaded onto a 10 ml Q Sepharose column. The enzyme was eluted from the column with a linear gradient of 0.2–0.6 M NaCl. Subsequent polyacrylamide gel electrophoresis of the pooled active protein peaks produced a single band, indicating a high degree of purity.

Cells of the *E. coli* XL1 strain harboring pVSEOP7 (our unpublished results) were used to produce the PTE enzyme. The pVSEOP7 plasmid contains a full-length *opd* gene cloned in the expression vector, pSE420 (Invitrogen Corp., CA, USA). The cells were grown in 11 batches to early log phase in LB broth in 61 Erlenmeyer flasks containing 100  $\mu$ g/ml ampicillin at 30 °C. After induction through the addition of IPTG to 0.6 mM, cells were grown an additional 14 h and cobalt chloride was added to 1 mM. Cells were harvested 4 h after cobalt addition, by centrifugation, and suspended in 10 mM bis-tris-propane, pH 7.8. Pellets

were frozen and stored at –40 °C before lysis by two passes through a French press. The cell-free extract supernatant was collected following centrifugation at 27,000 relative centrifugal forces for 45 min.

The native PTE enzyme was purified from the supernatant using a single strong cation exchange resin (SP-Sepharose). The fractions containing the PTE activity were pooled, concentrated, and dialyzed against 10 mM BTP, pH 7.8 containing 50  $\mu$ M cobalt chloride. The purified enzyme was analyzed through native acrylamide gel electrophoresis and estimated to be 85–90% homogeneous. The protein content was determined using Coomassie Protein Assay Reagent (Pierce, Rockford, IL, USA) using BSA as a standard protein.

## 3. Results

### 3.1. Assays—theory

Fluoride electrode assays offer a convenient means to determine an enzyme's relative activity on the different stereoisomers of an organophosphofluoridate substrate. If defluorination activity is approximately similar on all stereoisomers, the shape of the plot of free (released) fluoride versus time will approximate that obtained by base-mediated hydrolysis, which works equally on all isomers. If half the isomers are degraded significantly more rapidly than the others, there will be a midpoint deflection in the slope of the line when approximately half the initial substrate concentration has reacted.

### 3.2. *Alteromonas* sp. JD6.5 OPAA catalytic hydrolysis of racemic GF: activity at 3 mM versus activity at 0.5 mM

Data illustrated in Fig. 2 show the results of *Alteromonas* sp. JD6.5 OPAA catalysis of 3 mM GF. The monophasic curve is consistent with that of an enzyme that possesses sim-

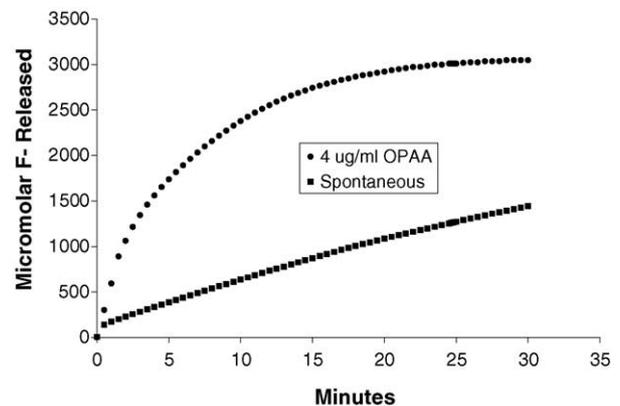


Fig. 2. Activity profile of the *Alteromonas* sp. JD6.5 OPAA enzyme with 3 mM GF. The monophasic curve is consistent with equal catalysis of both GF isomers.

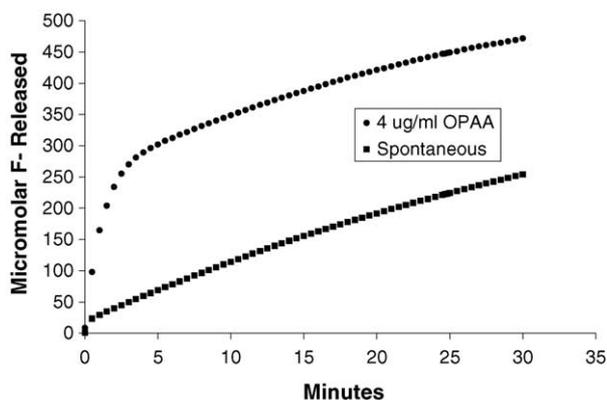


Fig. 3. Activity profile of *Alteromonas* sp. JD6.5 OPAA with 0.5 mM GF. The curve shows a distinct midpoint deflection, consistent with differential activity on the two isomers.

ilar activity on each of the two isomers (i.e., all the substrate is hydrolyzed at a uniform rate).

Fig. 3 shows the activity of *Alteromonas* sp. JD6.5 OPAA on 0.5 mM GF. At this concentration, the curve exhibits a distinct midpoint deflection with the slope of the second part of the curve approaching that of the spontaneous hydrolysis (i.e., half the substrate is hydrolyzed significantly more rapidly than the other half).

In 1965, Christen and Van den Muysenberg observed a biphasic hydrolysis of low GB concentrations in rat plasma [31]. When they did the same experiment with diisopropylfluorophosphate (a symmetrical molecule) the hydrolysis curve was monophasic. Also, when they did the experiment with higher GB concentrations the curve was monophasic. They hypothesized that free fluoride had catalyzed the racemization of the higher concentration of GB. This was supported by their observation that the addition of NaF to the plasma along with low concentrations of GB, again yielded a monophasic hydrolysis curve.

To determine if GF behaved similarly when its hydrolysis was catalyzed by *Alteromonas* sp. JD6.5 OPAA, NaF was added to a final concentration of 1 mM in the enzymatic reaction containing 0.5 mM GF. This addition of NaF caused a marked reduction in the midpoint deflection of the curve upon the addition of NaF to the reaction. The addition of NaCl to a final concentration of 1 mM in the same reaction caused no significant change in the reaction profile. These results (not shown) are consistent with fluoride-catalyzed racemization of GF.

### 3.3. Effects of GF concentration on stereospecificity

The effect of various higher GF concentrations up to 3 mM was examined in a series of reactions, each with 2  $\mu$ g/ml PTE. A gradual attenuation of the midpoint deflection in the curve was observed, with a corresponding increase in the starting substrate concentration. Results were again consistent with fluoride-catalyzed racemization of GF.

### 3.4. Gas chromatographic separation of GF isomers

In order to independently analyze the two GF stereoisomers, an isothermal gas chromatographic (GC) method was developed (Section 2.2). The two GF stereoisomers were consistently separated by 0.15–0.2 min at retention times between 8 and 9 min, depending on the flow rate of the carrier gas. When racemic GF was injected, the area of the first peak was always slightly greater than half the area of the second peak (Fig. 4a).

### 3.5. Enzymatic preparation and polarimetry analysis of a single (–)GF isomer

A chromatographically pure (–)GF isomer was prepared by specifically degrading the isomer for which the enzyme had the greater activity. The *Alteromonas* sp. JD6.5 OPAA enzyme reaction was conducted at 15 °C and pH 7.0 to minimize spontaneous hydrolysis. At a point slightly past the midpoint deflection in the reaction profile, the solution was extracted with dichloromethane and the extract was analyzed by GC. A single GF isomer peak was observed with a retention time corresponding to the second GC peak (Fig. 4b).

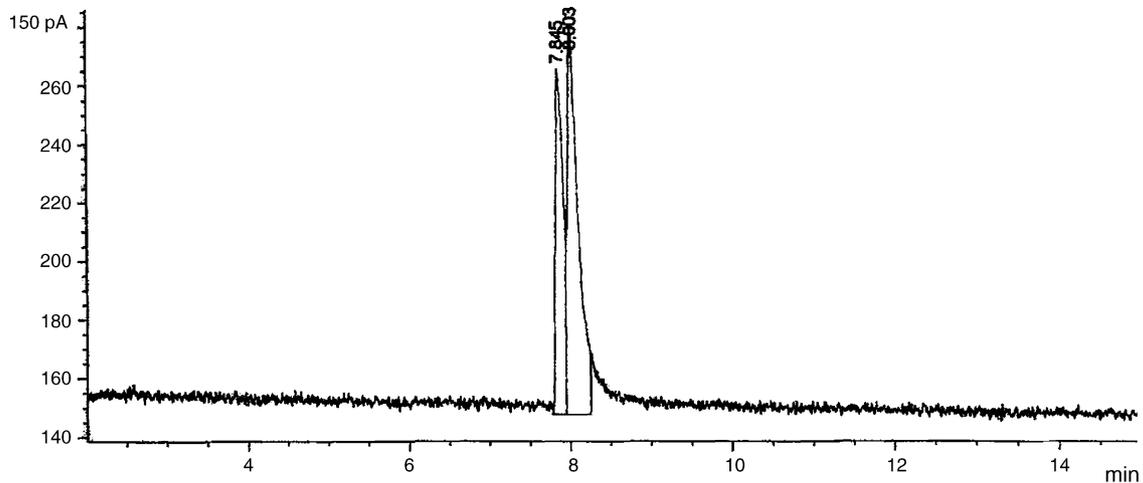
The single isomer preparation was concentrated approximately 10-fold by evaporation of the solvent at room temperature. The specific rotation was measured by polarimetry at –19.3°. Therefore, the enzymatic preparation was enriched for the (–)GF isomer, indicating the *Alteromonas* sp. JD6.5 OPAA enzyme specifically degraded the (+)GF isomer.

### 3.6. Fluoride-catalyzed racemization versus allosteric alteration of the enzyme

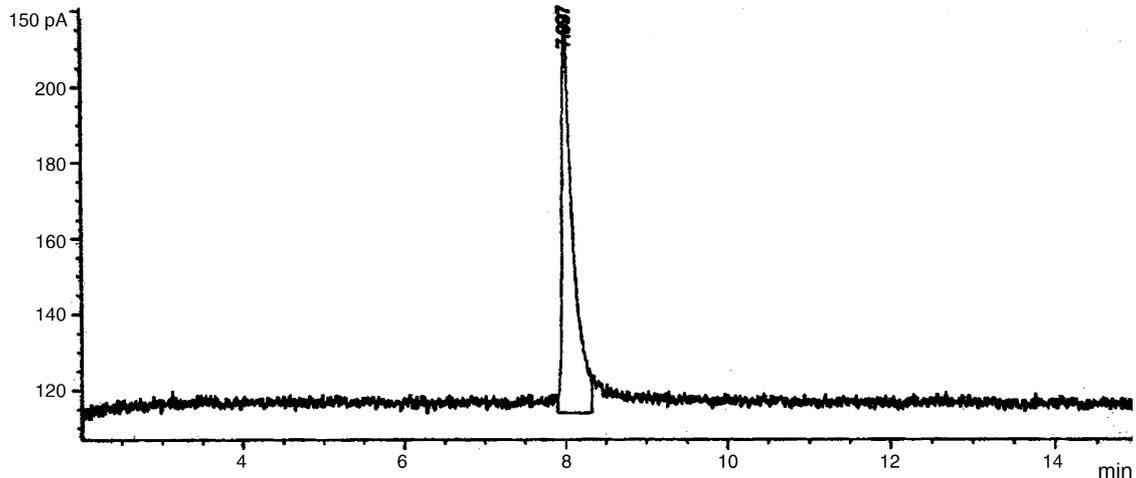
Although the results described above from the addition of NaF to the enzymatic reaction are consistent with fluoride-catalyzed racemization, an alternative explanation could be that fluoride was allosterically altering the enzyme and thereby changing its stereospecific properties. In order to determine if the racemization could proceed in the absence of enzyme, a solution of this single (–)GF isomer in dichloromethane was added at 50% volume to 2 M NaF, in the absence of enzyme. Subsequent GC analysis showed two peaks, consistent with NaF catalyzed non-enzymatic racemization of GF. Therefore, the presence of fluoride is both a necessary and sufficient condition for racemization, with or without enzyme.

### 3.7. Complementation test to determine stereospecificity of three enzymes

*Alteromonas* sp. JD6.5 OPAA, *A. haloplanktis* OPAA and wild-type PTE enzymes were tested individually and in combination to compare their stereospecificity on 0.5 mM GF. As shown in Fig. 5, the individual enzyme reactions all produced



Peak #	Ret Time	Area %
1	7.845	37.40245
(a) 2	8.003	62.59755



Peak #	Ret Time	Area %
(b) 1	7.997	1.000e2

Fig. 4. (a) Gas chromatogram of 90 °C isothermal separation of GF isomers. (b) GF following OPAA degradation of one isomer—only the second peak remains.

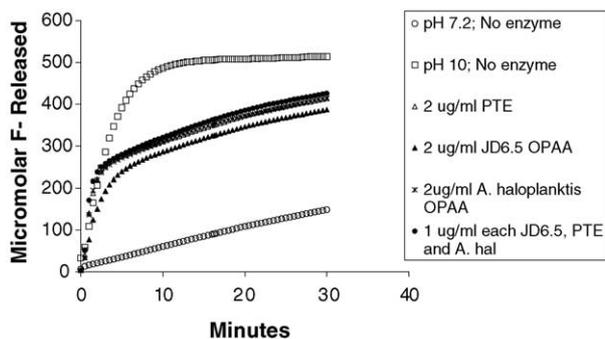


Fig. 5. Biphasic fluoride release with PTE, *Alteromonas* sp. JD6.5 OPAA and *A. haloplanktis* OPAA, each alone and in combination. No complementation was evident, indicating that all three enzymes exhibited a marked preference for the same GF stereoisomer.

similarly biphasic profiles. A complementation test was used to determine if these enzymes were all acting primarily on the same isomer or if one enzyme had primary activity on a different isomer than the other two. If any two enzymes tested in combination had significantly different stereospecificity, then the shape of the curve should have tended towards monophasic. Experimentally, the profile of the three-enzyme reaction was essentially indistinguishable from the individual enzyme reaction profiles. Lacking complementary activity, it was evident that all three enzymes were primarily active on the same isomer. Since the polarimetry data established that the preference of the *Alteromonas* sp. JD6.5 OPAA enzyme was for the (+)GF isomer, it was therefore concluded that the PTE and *A. haloplanktis* OPAA enzymes also exhibit preferential activity on the (+)GF isomer.

Table 2

Estimated specific activity of JD6.5 OPAA, *A. haloplanktis*, and PTE enzymes on (+) and (–) isomers of GF

Enzyme	(+) isomer	(–) isomer	Ratio (+)/(–)
PTE	66.5	3.1	21.5
JD6.5 OPAA	29.2	2.4	12.0
<i>A. haloplanktis</i>	65.3	2.7	24.3
PTE + JD6.5 OPAA + <i>A. haloplanktis</i> (1/3 each)	50.1	2.1	23.4

All values in  $\mu\text{mol}/\text{min}/\text{mg}$ .

### 3.8. Estimation of specific activity on each isomer

The rates in the first and second portion of the curves produced by each enzyme were calculated over a 1 min period and corrected for the spontaneous hydrolysis rate at a similar substrate concentration to yield an estimate of the relative activity ratios of the enzymes, alone and together, on each GF isomer. Results (Table 2) reflect a 12–24-fold difference in the activity estimates on the (+)GF and (–)GF isomers.

### 3.9. AChE inhibition of respective GF isomers

Since G-type chemical nerve agents exert their toxic effect through the inhibition of AChE enzymes, the in vitro measurement of that inhibition provides an indication of the expected in vivo toxicity. AChE inhibition experiments would be expected to indicate which, if either, of the two GF isomers contributed most strongly towards the known toxicity of the racemic compound. Given that the (–) isomers of GA, GB, GD and VX are all much more toxic than the (+) isomers (Table 1), the logical hypothesis is that the (–)GF isomer will inhibit AChE more strongly than racemic GF. If essentially all the toxicity is derived from the (–)GF isomer (as is the case with GB, for example), then the slope of the AChE inhibition with (–)GF should be twice as steep as the inhibition with racemic GF. On the other hand, if essentially all the toxicity of GF were derived from the (+) isomer, the (–)GF inhibition slope should be much less steep than with racemic GF. If the two GF isomers are of similar toxicity, the (–)GF slope and the racemic GF slope should be similar. The availability of enzymatic preparations of chromatographically pure (–)GF made it possible to compare of the AChE inhibition of this isomer to the racemic material to test the hypothesis above.

AChE inhibition was measured as a function of time in the presence of racemic GF, chromatographically pure (–)GF, and in the absence of GF. Fig. 6 shows the results of the AChE inhibition assays. All assays were performed in triplicate and the error bars represent the standard deviations of the three assays for each point. Clearly, the (–)GF inhibits the AChE much more strongly than the racemic GF, and therefore represents the more toxic isomer. These data, in combination with the results of the complementation experiment, indicate that all three wild-type bacterial enzymes tested preferentially degrade the (+)GF isomer, which has the lowest AChE binding activity of the two isomers.

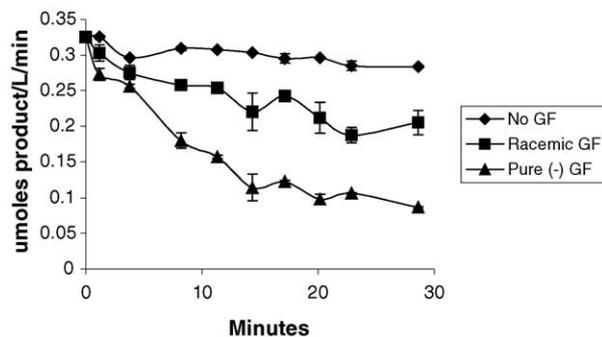


Fig. 6. AChE inhibition by racemic GF and chromatographically pure (–)GF.

### 3.10. Stereospecificity of the PTE mutant H254G/H259W/L303T (GWT)

In an attempt to identify an enzyme with preferential activity on the (–)GF isomer, a number of PTE mutants were screened. Most of these mutants showed the same preference as the wild-type enzyme or had very low levels of activity overall. The PTE mutant H254G/H259W/L303T (GWT) was of particular interest, since that mutant's stereospecificity toward *p*-nitrophenyl derivatives of G-type nerve agents was known to be reversed from that of the wild-type enzyme (*p*-nitrophenyl derivatives have the fluoride leaving group of G-agents substituted with a *p*-nitrophenyl leaving group to allow for colorimetric detection of the hydrolysis product) [28]. Fig. 7 shows the GF hydrolysis profiles for the GWT mutant and *Alteromonas* sp. JD6.5 OPAA individually, and for the two enzymes combined. Each enzyme alone exhibits a midpoint deflection but the combination of the two enzymes clearly shows complementation (monophasic catalysis). Therefore, both enzymes exhibit stereospecificity and their stereospecificity is opposite each other, meaning the GWT mutant is preferentially catalyzing hydrolysis of the (–)GF isomer. The specific activity on each isomer was estimated as in Section 3.8. The specific activity from the first portion of the curve (corresponding primarily to catalysis of the (–) isomer) was estimated at  $2.9 \mu\text{mol}/\text{min}/\text{mg}$  and from the second portion of the curve was estimated at  $0.5 \mu\text{mol}/\text{min}/\text{mg}$ . Therefore, compared to the values for the native enzymes shown in Table 2, the activity on the (–) isomer has changed very little while the activity on the (+) isomer has been reduced by approximately two orders of magnitude. The estimated (+)/(–) activity ratio of the mutant enzyme is 0.16, as compared to 21.5 for the native PTE, indicating a reversal of stereospecificity as compared to wild-type.

### 3.11. Enzymatic preparation and polarimetry analysis of a single (+)GF isomer

A chromatographically pure (+)GF isomer was prepared by specifically degrading the (–) isomer, on which the GWT enzyme had the greater activity. Slightly past the midpoint

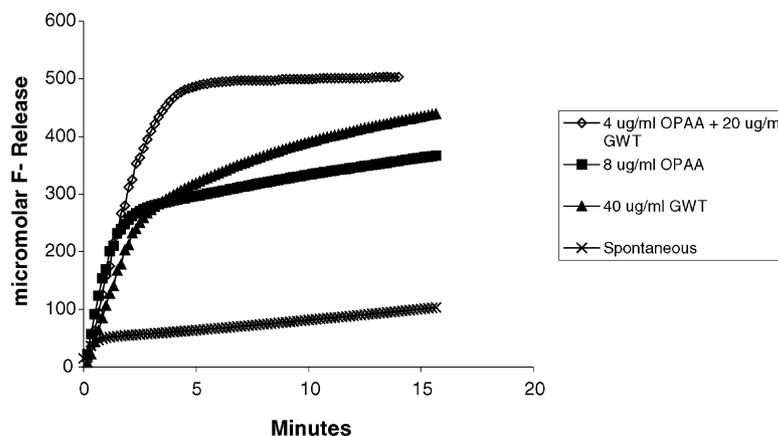


Fig. 7. Complementation of GF stereochemistry: PTE mutant H254G/H259W/L303T (GWT) and *Alteromonas* sp. JD6.5 OPAA. Enzyme concentration of *Alteromonas* sp. JD6.5 OPAA is five-fold lower than GWT due to its overall higher level of activity.

deflection of the reaction profile, the solution was extracted with dichloromethane and the extract was analyzed by GC. In the chromatogram, a single GF isomer peak was observed with a retention time corresponding to (+)GF. Results are shown in Fig. 8a (racemic GF control) and Fig. 8b (enzymatic preparation).

The single isomer preparation was concentrated approximately 10-fold by evaporation at room temperature and the specific rotation was measured at  $+5^\circ$ . Therefore, the enzymatic preparation was enriched for the (+)GF isomer, further confirming that the GWT enzyme specifically degraded the (–)GF isomer.

#### 4. Discussion

The data presented here demonstrate for the first time the stereospecificity of the *Alteromonas* sp. JD6.5 OPAA, *A. haloplanktis* OPAA and the PTE enzymes (including the GWT mutant) in the catalytic hydrolysis of GF. Complementation assays specifically showed the result of protein engineering of the PTE enzyme to reverse the stereospecificity and accomplish the preferential degradation of the (–)GF isomer, which was the isomer shown here to possess the greatest AChE-inhibiting activity. Given that almost all the toxicity of GA, GB, GD and VX is derived from the (–) isomers, and that the AChE-inhibition of the (–) GF isomer was about twice that of racemic GF, it is evident that the (–)GF isomer contributes most, and possibly almost all, of the toxicity to the racemic compound. This conclusion would also be consistent with basic expectation given the consistency of the isomer toxicity differences throughout the range of AChE-inhibiting compounds shown in Table 1.

Stereochemical variations in biologically active molecules often play a major role in the effectiveness of drugs or the specificity of pesticides. Toxin molecules such as Botulinum toxin and other toxins fused to antibodies are used as therapeutics. One means of the preparation of pure stereoisomers is through the preferential enzymatic degradation of one

stereoisomer, leaving most of the other isomer intact. In these investigations, the stereospecificity of the wild-type enzymes permitted the preparation of a pure sample of (–)GF by preferentially degrading the (+)GF stereoisomer and extracting the resulting product. Polarimetry established the identity of the remaining isomer, which then allowed the assignment of peaks in the isothermal GC method that was developed to resolve the two GF isomers and permit their chromatographic identification either alone or together. A chromatographically pure preparation of (+)GF was made using the GWT mutant of PTE to preferentially degrade the (–) stereoisomer. Polarimetry analysis confirmed the chiral identity of the extracted and concentrated product. Thus, preparations of either GF isomer could be made from the racemic mixture by using the enzyme with the corresponding stereospecificity.

The enzyme-catalyzed hydrolysis of G-type chemical nerve agents has been investigated for several different potential applications, including surface or personnel decontamination and the use of encapsulated enzymes as *in vivo* scavengers or catalytic treatments for nerve agent poisoning. For instance, the protection of mice from organophosphate intoxication was strikingly enhanced when *in vivo* circulating encapsulated enzymes were used in conjunction with 2-pyridinealdoxime methyl chloride (2-PAM) and/or atropine [32]. For this application, it is important to consider that toxic concentrations of agent in the blood would be orders of magnitude below the highest concentrations at which stereospecificity is observed. Therefore, it would be critical to use enzymes that are active on the toxic isomers at these low concentrations.

When used on higher concentrations of agent such as could be encountered in surface decontamination applications, the fluoride product, which was shown here to be a necessary and sufficient condition for GF racemization, might catalyze racemization of the substrate and thereby facilitate complete degradation of the contaminant. Alternatively, an aqueous decontamination solution could be augmented with millimolar concentrations of NaF to ensure the racemization reaction occurs.

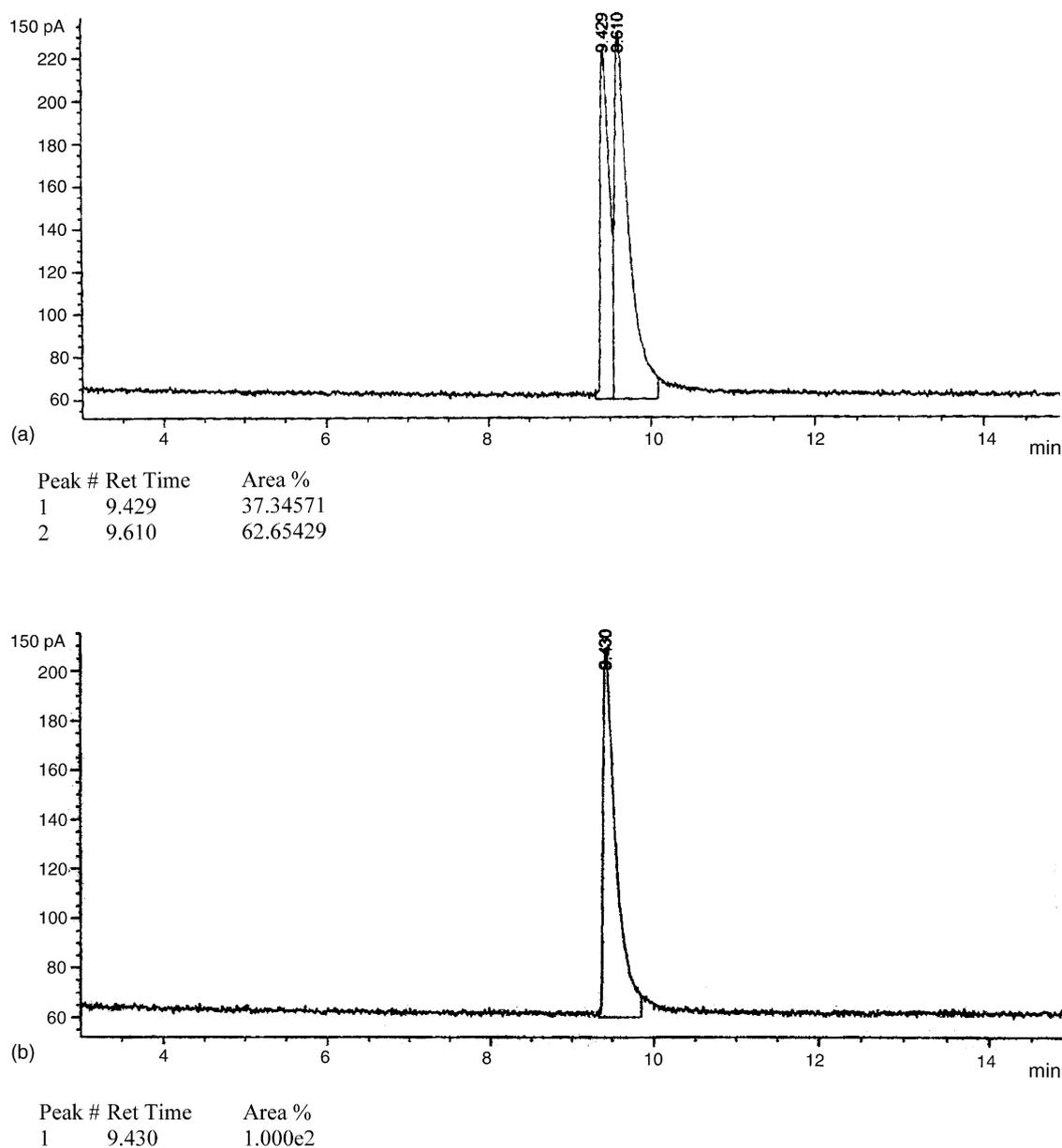


Fig. 8. (a) Racemic GF prior to enzymatic catalysis. Peak #1 is (+)GF and peak #2 is (–)GF. (b). GF following enzymatic degradation of one isomer with the PTE mutant H254G/H259W/L303T. Note that only peak #1 remains, indicating the preferential catalysis of (–)GF.

There are also some highly toxic agents such as GV (2-dimethylaminoethyl *N,N*-dimethylphosphonamido-fluoridate) that are largely refractory to traditional atropine/reactivator treatments [33]. In these cases, enzymes offer a potential alternative treatment regime. In the cases where these agents have chiral centers, it will be important to evaluate the enzyme activity on all isomers, particularly those with the greater toxicity.

#### Acknowledgments

We are grateful to Dr. Diane Dutt for her thorough critique of this manuscript and to the National Institutes of Health

(GM 68550) and U.S. Army Medical Research and Materiel Command (DAMD17-00-2-0010) for financial support.

#### References

- [1] DeFrank JJ, Beaudry WT, Cheng T-C, Harvey SP, Stroup AN, Szafraniec LL. Screening of halophilic bacteria and *Alteromonas* species for organophosphorus hydrolyzing enzyme activity. *Chem Biol Interact* 1993;87:414–48.
- [2] DeFrank JJ, Cheng T-C, Kolakowski JE, Harvey SP. Advances in the biodegradation of chemical warfare agents and related materials. *J Cell Biochem Suppl* 1995;0(21A):41.
- [3] Cheng T-C, Harvey SP, Chen GL. Cloning and expression of a gene encoding a bacterial enzyme for decontamination of organophos-

- phorus nerve agents and nucleotide sequence of the enzyme. *Appl Environ Microbiol* 1996;62:1636–41.
- [4] Cheng T-C, Liu L, Wang B, DeFrank JJ, Anderson DM, Hamilton AB. Nucleotide sequence of a gene encoding an organophosphorus nerve agent degrading enzyme from *Alteromonas haloplanktis*. *J Ind Microbiol Biotechnol* 1997;18:49–55.
- [5] Mulbry WW, Karns JS, Kearney PC, Nelson JO, McDaniel CS, Wild JR. Identification of a plasmid-borne parathion hydrolase gene from *Flavobacterium* sp. by Southern hybridization with *opd* from *Pseudomonas diminuta*. *Appl Environ Microbiol* 1986;51:926–30.
- [6] McDaniel CS, Harper LL, Wild JR. Cloning and sequencing of a plasmid-borne gene (*opd*) encoding a phosphotriesterase. *J Bacteriol* 1988;170:2306–11.
- [7] Serdar CM, Murdock DC, Rohde M. Parathion hydrolase gene from *Pseudomonas diminuta* MG: subcloning, complete nucleotide sequence, and expression of the mature portion of the enzyme in *Escherichia coli*. *Bio/Technology* 1989;7:1151–5.
- [8] Serdar CM, Gibson DT. Enzymatic hydrolysis of organophosphates: cloning and expression of a parathion hydrolase gene from *Pseudomonas diminuta*. *Bio/Technology* 1985;3:567–71.
- [9] Dave KI, Miller CE, Wild JR. Characterization of organophosphorus hydrolases and the genetic manipulation of the phosphotriesterase from *Pseudomonas diminuta*. *Chem Biol Interact* 1993;87:55–68.
- [10] Phillips JP, Xin JH, Kirby K, Milne CP, Krell P, Wild JR. Transfer and expression of an organophosphate insecticide-degrading gene from *Pseudomonas melanogaster*. *Proc Natl Acad Sci USA* 1990;87:8155–9.
- [11] Rowland SS, Speedie MK, Pogell BM. Purification and characterization of a secreted recombinant phosphotriesterase (parathion hydrolase) from *Streptomyces lividans*. *Appl Environ Microbiol* 1991;57:440–4.
- [12] Dumas DP, Caldwell S, Wild JR, Raushel FM. Purification and properties of the phosphotriesterase from *Pseudomonas diminuta*. *J Biol Chem* 1989;264:19659–65.
- [13] Dumas DP, Durst HD, Landis WG, Raushel FM, Wild JR. Inactivation of organophosphorus nerve agents by the phosphotriesterase from *Pseudomonas diminuta*. *Arch Biochem Biophys* 1990;277:155–9.
- [14] Ashani Y, Shapira S, Levy D, Wolfe AD. Butyrylcholinesterase and acetylcholinesterase prophylaxis against soman poisoning in mice. *Biochem Pharmacol* 1991;41:37–41.
- [15] Kolakowski JE, DeFrank JJ, Harvey SP, Szafraniec LL, Beaudry WT, Lai K, et al. Enzymatic hydrolysis of the chemical warfare agent VX and its neurotoxic analogues by organophosphorus hydrolase. *Biocatal Biotransform* 1997;15:297–312.
- [16] Harvey SP. Enhancement of the GD catalytic activity of the OPH enzyme by protein engineering: screening and kinetic analysis of two mutants. ERDEC-TR-492, April 1998.
- [17] Harvey SP, Kolakowski JE, DeFrank JJ, Chen-Goodspeed M, Soborb M, Raushel FM. Increased VX and G-Agent activity of various phosphotriesterase enzyme mutants. ECBC-TR-188, September 2001.
- [18] Lai K, Grimsley JK, Kuhlmann BD, Scapozza L, Harvey SP, DeFrank JJ, et al. Rational enzyme design: computer modeling and site-directed mutagenesis for the modification of catalytic specificity in organophosphorus hydrolase. *Chimia* 1996;50(9):430–1.
- [19] Michel HO. Kinetics of the reactions of cholinesterase, chymotrypsin and trypsin with organophosphorus inactivators. *Fed Am Soc Exp Biol* 1955;14:255.
- [20] Boter HL, van Dijk C. Stereospecificity of hydrolytic enzymes in reaction with a symmetric organophosphorus compound: the inhibition of acetylcholinesterase and butyrylcholinesterase by enantiomeric forms of sarin. *Biochem Pharmacol* 1969;18:2403–7.
- [21] Van De Meent, et al., TNO, Unpublished results, 1987.
- [22] Hall CR, Inch TD, Inns RH, Muir AW, Sellers DJ, Smith AP. Differences between some biological properties of enantiomers of alkyl S-alkyl methylphosphonothioates. *J Pharm Pharmacol* 1977;29:574–6.
- [23] Benschop HP, Konings CA, Van Genderen J, De Jong LP. Isolation, anticholinesterase properties, and acute toxicity in mice of the four stereoisomers of the nerve agent soman. *Toxicol Appl Pharmacol* 1984;72:61–74.
- [24] Degenhardt CE, Van den Berg GR, De Jong LPA, Benschop HP, Van Genderen J, Van de Meent D. Enantiospecific complexation gas chromatography of nerve agents: isolation and properties of the enantiomers of ethyl N,N-dimethylphosphoramidocyanidate (tabun). *J Am Chem Soc* 1986;108:8290–1.
- [25] Material Safety Data Sheet for GF. Edgewood Arsenal Special Report EO-SR-74002, December 1974. Available from Defense Technical Information Center as AD C014 792.
- [26] Ellman GL, Courtney KD, Andres Jr V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 1961;7:88–95.
- [27] Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 1989;77:51–9.
- [28] Hill CM, Li WS, Thoden JB, Holden HM, Raushel FM. Enhanced degradation of chemical warfare agents through molecular engineering of the phosphotriesterase active site. *J Am Chem Soc* 2003;125:8990–1.
- [29] Cheng T-C, DeFrank JJ, Rastogi VK. *Alteromonas* prolidase for organophosphorus G-agent decontamination. *Chem Biol Interact* 1999;119/120:455–62.
- [30] Cheng T-C, Liu L, Wang B, Wu J, DeFrank JJ, Anderson D, et al. Nucleotide sequence of a gene encoding an organophosphorus nerve agent degrading enzyme from *Alteromonas haloplanktis*. *J Ind Microbiol* 1997;18:49–55.
- [31] Christen PJ, Van den Muysenberg JA. The enzymatic isolation and fluoride catalysed racemisation of optically active sarin. *Biochim Biophys Acta* 1965;220:217–20.
- [32] Petrikovic I, Cheng T-C, Papahadjopoulos D, Hong K, Yin R, DeFrank JJ, et al. Long circulating liposomes encapsulating organophosphorus acid anhydrolase in diisopropylfluorophosphate antagonism. *Toxicol Sci* 2000;57:16–21.
- [33] Bajgar J, Fusek J, Hrdina V, Patocka J, Vachek J. Acute toxicities of 2-dialkylaminoalkyl-(dialkylamido)fluorophosphates. *Physiol Res* 1992;41:399–401.